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Are the leading drugs against *Staphylococcus aureus* really toxic to cartilage?☆

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Summary Many studies have shown that the toxic effects of local antibiotics on bone and cartilage limit orthopedic surgeons. In this study, we evaluated three antibacterial agents used locally to treat highly mortal and morbid diseases in the field of orthopedics, such as septic arthritis. Are vancomycin, teicoplanin, and linezolid, which are archenemies of *Staphylococcus aureus*, really toxic to chondrocytes? The purpose of the study was to investigate the effects of antibiotics, which are used against *S. aureus*, on human chondrocytes in vitro.

Primary cell cultures obtained from gonarthrosis patients were divided into two main groups. One of these groups was designated as the control chondrocyte culture. The other group was divided into three subgroups, and each group was exposed

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to vancomycin, teicoplanin, or linezolid. Cell culture samples were characterized by immunophenotyping following incubation with the three different antibiotics. Before and after the agents were administered, the cultures were subjected to inverted and environmental scanning electron microscopy. The number of live cells and the proliferation rate were monitored with the MTT-assay.
We found that vancomycin, teicoplanin, and linezolid do not have chondrotoxic effects.
Vancomycin, teicoplanin, and linezolid had no chondrotoxic activity during in vitro culture, which supports the argument that these agents can safely be used in orthopedic surgery, especially against methicillin-resistant *S. aureus* agents.

Introduction

Side effects and adverse reactions occur frequently during systemic antibiotic therapies [1,2]. The prolonged and high-dose administration of antibiotics required to treat septic arthritis and osteomyelitis results in side-effect profiles that also involve toxicity problems [1,3—5]. To avoid such deleterious effects, local antibiotics are often utilized, especially intra-operatively [6—11]. In orthopedics, placing antibiotic-impregnated Spongostan or gels/grafts in various forms into infected regions has been found useful to fight infection or to act as a prophylactic [2,3,7—9]. However, the toxicity of these local antibiotics has been discussed in the literature [1]. Additionally, reports indicating local osteotoxic and chondrotoxic effects have imposed some limitations on orthopedic surgeons [1,10], especially with regard to certain procedures involving open surgeries, such as intra-articular fracture surgery and multiple ligament knee injuries. In such cases, both prophylaxis and the use of antibiotic therapy may be helpful [12,13]. In the present study, vancomycin (VCM) [6], known to be chondrotoxic, and its alternatives teicoplanin (TEIC) and linezolid (LZD), commonly used against *Staphylococcus aureus*, the most common cause of septic arthritis, were specifically chosen due to their common use in orthopedics. The chondrotoxicity of these three antibiotics are investigated in human primary chondrocyte cultures.

Materials and methods

Prior to the present research, approval from the Namik Kemal University Medical Faculty Ethics Committee (Date: 25/12/2014; Number: 127) and the participant written informed consents were obtained. Primary chondrocyte cultures consisted of surgically harvested osteochondral tissues from gonarthrosis patients undergoing routine total knee arthroplasty, ($n = 6$); VCM, TEIC, and LZD agents were added, and cell viability, toxicity, and proliferation analyses were conducted at baseline (0 h), before the application of antibiotics, and after 24 h. Surface morphologies and the extracellular matrix were examined using inverted and environmental scanning electron microscopes (ESEM). To minimize measurement and analysis errors, the same researcher performed the analyses both during and after the primary chondrocyte culturing. The researchers conducting the analyses were blinded to the type of drug used and the experimental setup. All experiments were conducted three times.

Materials

Collagenase type II enzyme (1 mg/mL, Invitrogen Corporation); Hank's Balanced Salt Solution (HBSS-1X, 14025, Gibco); penicillin-streptomycin with fetal bovine serum (FBS); and Dulbecco's Modified Eagle's Medium (DMEM) (1000 mg glucose/L) were obtained from Sigma Chemical, St. Louis, USA. Sodium dodecyl sulfate (SDS) (10% L4522), insulin—transferrin—selenium (ITS) premix, and RPMI-1640 were obtained from Sigma—Aldrich GmbH, Germany. The MTT Kit was a Vybrant MTT Cell Proliferation Assay (Catalog ≠ V-13154) by Cell Biolabs Inc., USA. A laminar flow cabinet (Air Flow-NUVE/NF-800 R) and an incubator (NUVE, 06750) were obtained in Ankara, Turkey. An Olympus CKX41 inverted microscope was used and the images were recorded using an Olympus cell soft imaging system program. A Mindray MR 96A (PRC) enzyme-linked immunosorbent assay (ELISA) device measured viability, cytotoxicity, and proliferation with a commercial kit. This study used a Quanta 250 FEG (Fei Company, Hillsboro, Oregon, USA) ESEM.

Methods

Patient selection criteria

Osteochondral tissues to be used in the primary chondrocyte culture were obtained from patients who were not allergic to VCM, LZD, or TEIC. Additionally, the study excluded patients with Parkinson's disease who had been treated with monoamine oxidase inhibitors, such as rasagiline, selegiline, or moclobemide, in the previous 14 days, as well as patients receiving antidepressant drugs, to prevent drug interaction. Linezolid is also one a monoamine oxidase inhibitor itself. Therefore, it can cause an increase in the adrenergic pressor response.

Preparation of the drugs

Main stock solutions were freshly prepared by dissolving VCM and TEIC in 1000 and 400 mg DMEM culture media, respectively, in a flow cabin. The LZD was prepared using a readymade 300 mL solution with a concentration of 2 mg/mL. Next, these main stock solutions were coded to keep the researchers blinded to the treatment before being transferred to dark-brown bottles.

Final concentrations of the drugs administered to the chondrocyte cultures were determined in relation to their minimal bactericidal concentrations (MBC). The practiced MBC reference values were 16 mg/L for VCM, 64 mg/L for TEIC, and 32 mg/L for LZD [14].

Obtaining the primary chondrocyte culture

The study included tissues obtained from gonarthrosis patients whose medical and conservative treatments had failed and who were undergoing total knee arthroplasty surgery [$n = 6$; mean age 65 years (59—71); Stage 4 gonarthrosis]. Patient informed consent was obtained. Osteochondral tissues were resected from the distal femur and the proximal tibia, which are routinely cut away during total knee arthroplasty. Standard human primary chondrocyte cultures were then performed [15—18].

The cells were counted on a Thoma counting chamber in trypan blue and placed in a 24-well plate with 125×10^3 cells in each well; they were then stored in an incubator for 48 h. At the end of this time period, the drugs were introduced into the cell cultures, which became confluent and stuck to the bottom.

Exposure of the drugs into the cultures

The control group, named Group I, consisted of pure primary chondrocyte cultures. Groups II, III, and IV consisted of primary chondrocyte cultures that contained VCM, LZD, and TEIC, respectively. An additional sample was kept aside from both the study and control groups for MTT-ELISA and ESEM analyses.

Analyses

Cells that displayed chondrotoxic activity and stuck to the inner surface of the flasks were examined under an inverted microscope, and the micro images were recorded. Inverted microscopy, MTT-ELISA cell viability and toxicity, and ESEM analyses were performed on Group I at both 0 and 24 h. The same analyses were performed on Groups II, III, and IV at 0 and 24 h after introducing the antibacterial agents.

ESEM analysis. In this stage, the surface topography and the extracellular matrix structures of the chondrocytes were examined, and the cell-culture contents of the wells were expelled using gun pipettors. A 2.5% glutaraldehyde solution composed of 97.5 mL cacodylate buffer and 2.5 mL glutaraldehyde was then added to the wells to cover the samples. The glutaraldehyde solution was removed from the samples using a pipette gun and kept at room temperature for 2 h. The samples were then washed three times with cacodylate buffer. Following the last washing, the samples were covered with cacodylate buffer and kept at 4 °C until analysis.

The MTT cell viability, toxicity, and proliferation assays. Viability tests were performed using a commercial MTT kit (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MW = 414) and following the manufacturer's instructions. The kit works as follows: mitochondrial dehydrogenase enzymes cleave to the tetrazolium ring, yielding blue formazan crystals, which do not occur in dead cells. A 12 mM MTT stock solution was prepared by dissolving 5 mg MTT in sterile phosphate buffer saline. The antibiotic medium was removed from the culture medium and the MTT medium was added to reach a final concentration of 10% (v/v). This mixture was incubated at 37 °C for 2 h. At the end of the incubation period, 500 μL DMSO were added as a solvent and the mixture was incubated again at 37 °C for 10 min. For each sample, spectrophotometry was performed at 540 nm to measure the absorbance. The project coordinator transcribed letter labels designated for each sample. The vitality of the control group was assumed to be 100% before the transfer of the antibiotics (0 h) to the culture medium. Twenty-four hours after the administration of the antibiotics, the viability absorbance of the cells was recorded in nanometers. Proliferation analyses for 24—36 h were then recorded.

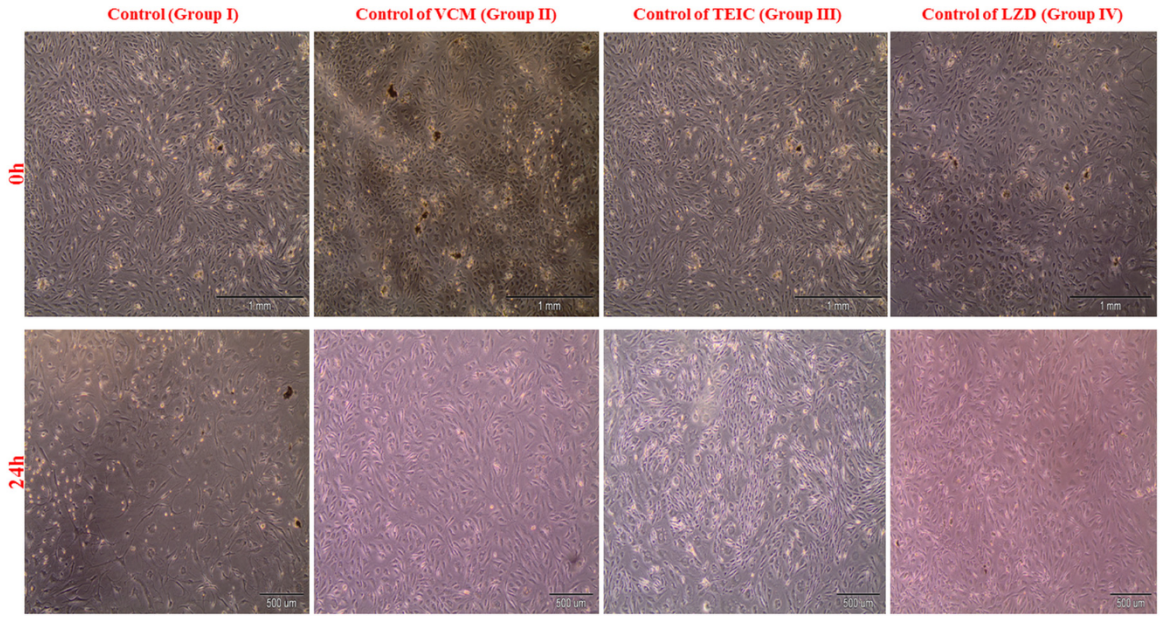

Figure 1 Invert microscopy images of chondrocytes at 0 and 24 h in the control group.

Statistical analysis. Cell numbers and proliferation results, along with Minitab R15 software, were used to analyze the data. Each drug was compared with its own control group using the variance analysis, and the proliferation data were analyzed using Tukey's Method (with 95.0% confidence intervals). The statistical significance level was set at $\alpha < 0.05$.

Findings

Analysis of inverted microscopy results

Images of proliferated healthy chondrocytes (91—99%) in the control group, which did not receive antibiotics (Fig. 1).

The statistical evaluation of the effects of the agents on chondrocytes via MTT ELISA/MTT-proliferation assays

The alive cell counts in all of the groups that received antibiotics were similar with respect to the control groups at 24 h. All three antibiotics continued to proliferate in the 36 h of test that began at the end of 24th hour (Fig. 2).

The 24 h viability and 36 h proliferation test results showed that there was no statically difference between the control groups and the groups that received antibiotics. All three agents were not toxic to chondrocytes (Table 1 and Fig. 3).

An evaluation of the ESEM analysis

Healthy cells and extracellular structures were seen in the morphological evaluations of the chondrocyte surfaces before antibiotic exposure (Fig. 4). ESEM images after 24 h of antibiotic exposure support the results of the MTT-ELISA viability, toxicity, and MTT-proliferation data analyses as well as the invert images (Fig. 5). Healthy chondrocytes and extracellular matrices, which were the markers of chondroblastic activity, and natural surface characteristics were visible in the images from all of the groups.

Table 1 Cartilage cells growth assessed by the MTT viability, toxicity, and proliferation assays (540 nm) after exposure to three different antibiotics.

Groups	Mean ± SD	p^a
MTT cell viability and toxicities		
Control of VCM	0.28200 ± 0.01699	
VCM	0.2735 ± 0.0594	0.743
Control of TEIC	0.2240 ± 0.0288	
TEIC	0.2362 ± 0.0752	0.719
Control of LZD	0.2653 ± 0.0482	
LZD	0.2705 ± 0.0540	0.865
MTT cell proliferation test		
VCM	107.4 ± 35.5A[b]	
TEIC	105.8 ± 28.3A[b]	0.914
LZD	101.63 ± 2.08A[b]	

[a] Analysis of variance test.
[b] Grouping information using Tukey's Method and 95.0% confidence.

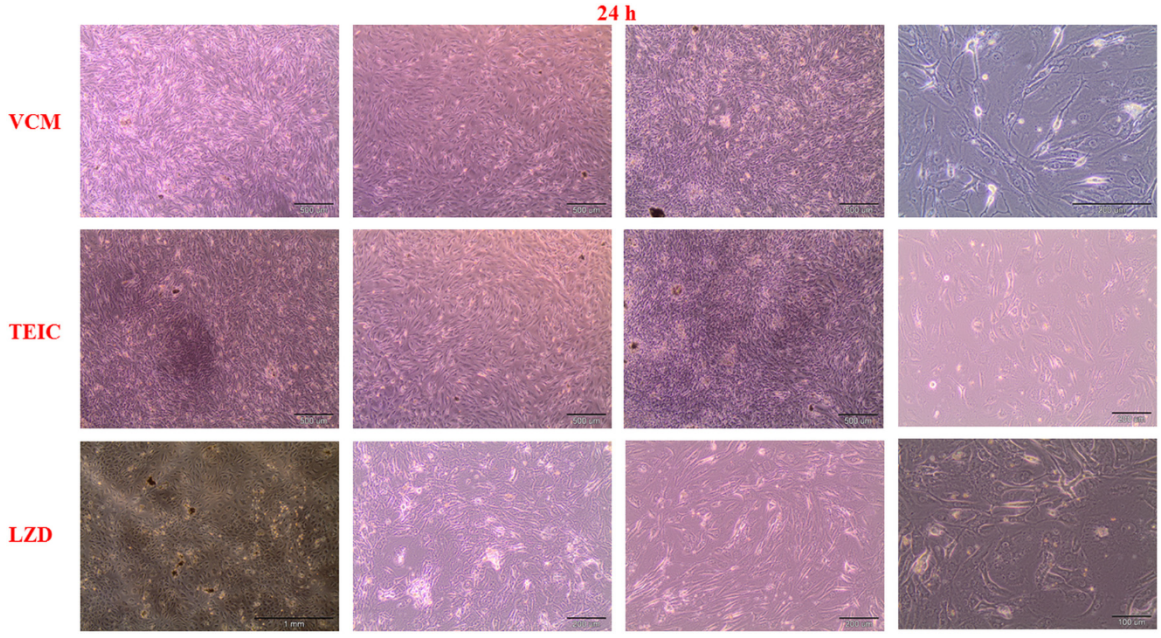

Figure 2 Invert microscopy images of chondrocytes after 36 h (the 24th hour proliferation test) of exposure to VCM, TEIC, and LZD.

Discussion

Orthopedic infections, which occasionally jeopardize the outcome of the surgery, usually require long-term antibiotic treatments. This molecular study investigated the cytotoxicity of VCM, TEIC, and LZD in human primary chondrocyte cultures. These agents were selected because they can be administered locally and systemically to treat methicillin-resistant *S. aureus* (MRSA), one of the leading causes of orthopedic infections.

Earlier studies claimed that VCM is chondrotoxic [6]. In addition to its systemic use, it is also administered locally to joints [3]. Studies comparing joint and serum levels following the intra-articular and parenteral administration of VCM concluded

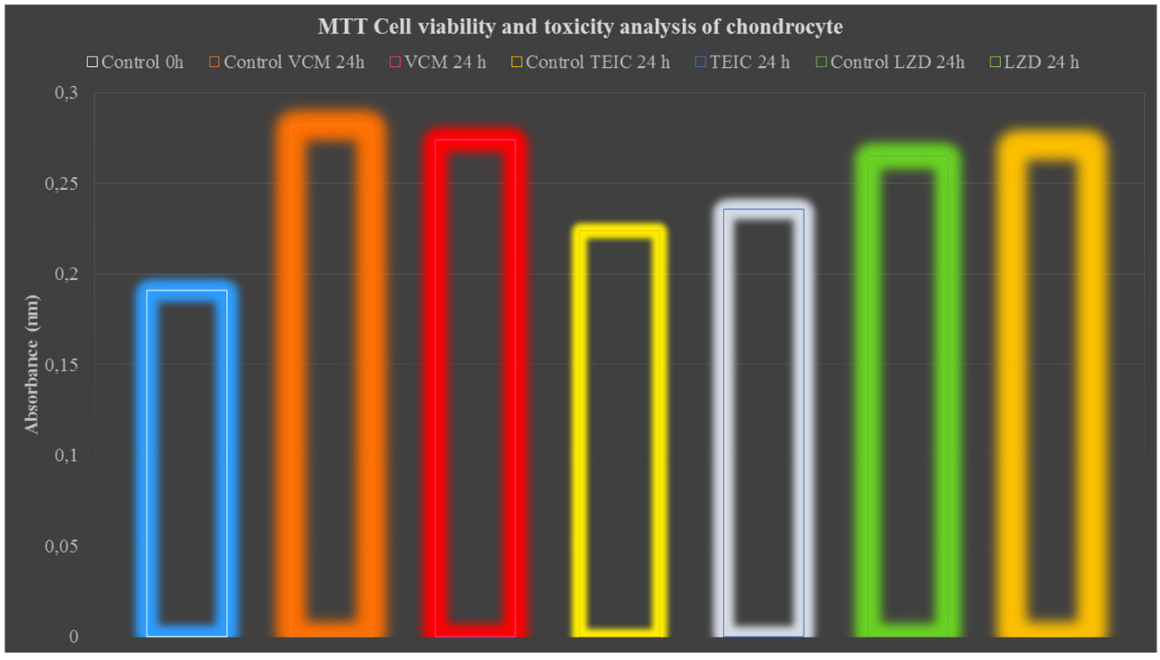

Figure 3 The absorbance of the live cell count after MTT-ELISA analyses in all groups.

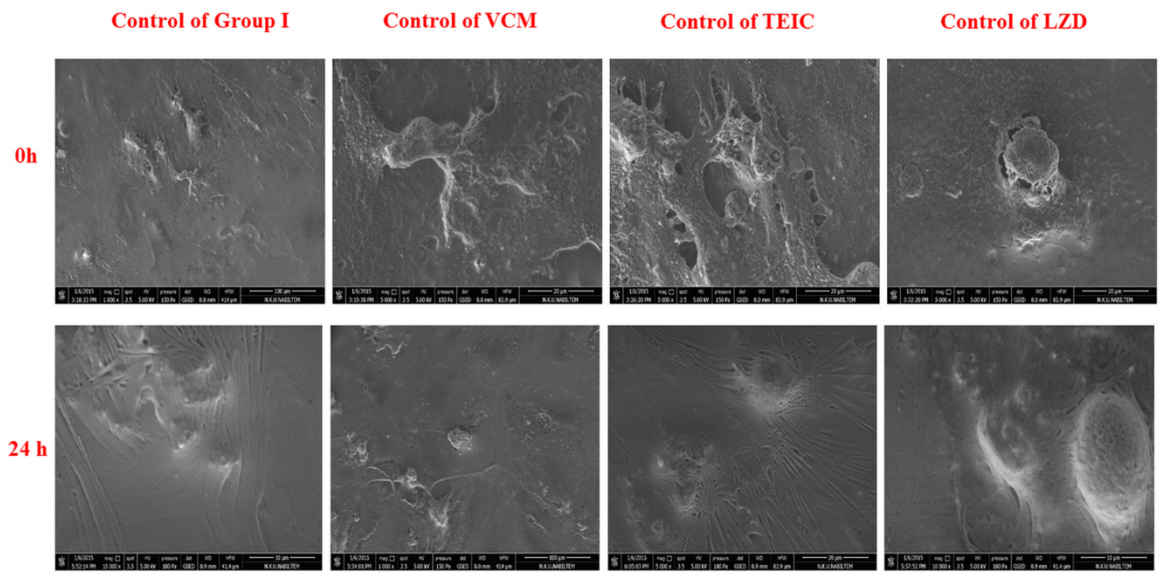

Figure 4 Morphological evaluations of chondrocyte surfaces in the control group (ESEM images at the 0 and 24th hour.

that intra-articular administration is more effective [19]. An in vivo study using a murine bacterial arthritis model reported that VCM and LZD can safely treat MRSA based on the findings that reducing inflammatory cytokines in the group treated with VCM and LZD led to decreased accumulated neutrophils [20].

Previous cytotoxicity studies investigated the chondrotoxicity of not only various antibacterial agents of the glycopeptide group but also other antibiotic agents, such as the quinolone group [21—23]. These studies examined the antibacterial agents used in orthopedic surgeries and the chondrotoxicity of a wide range of pharmaceutical agents, such as local anesthetics, opioids, and corticosteroids, on a molecular level [18,24,25].

Previous in vitro studies reported that VCM causes less osteoclast activity compared to tobramycin, cefazolin, and gentamicin. Moreover, cell-line comparisons showed that VCM in 125 g/mL doses is toxic to osteoblast- and chondroblast-like cells [1,6,26,27].

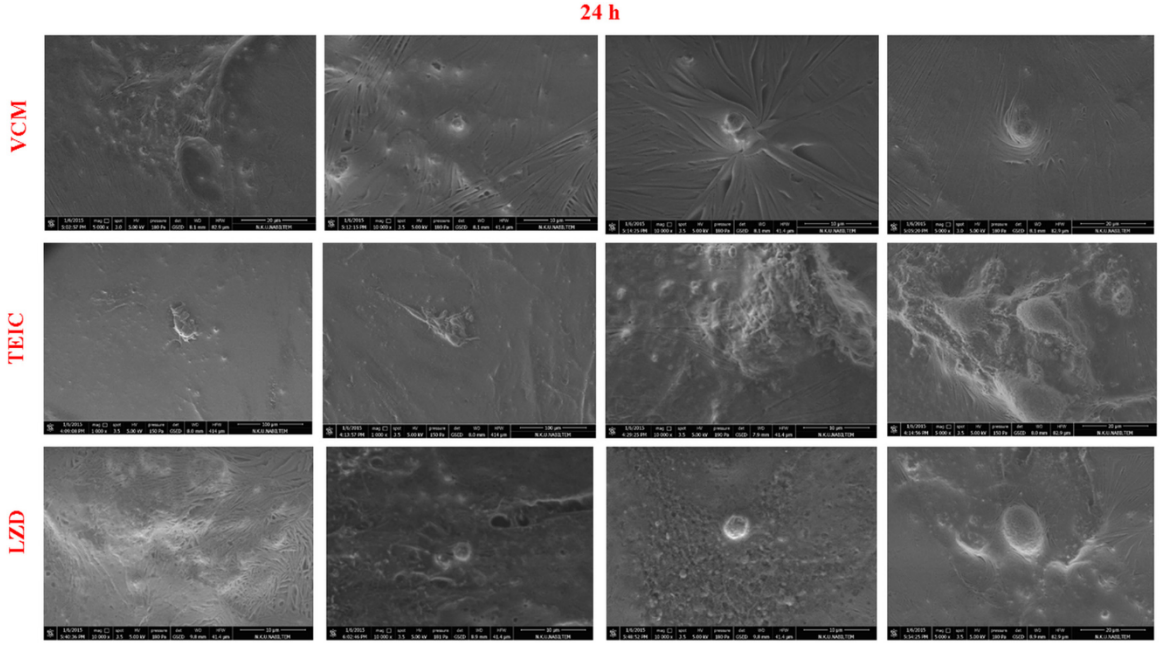

Figure 5 ESEM images at the 24th hour after the administration of antibiotics in primary human chondrocytes.

Antoci et al. investigated the chondrotoxicity of the glycopeptide group in cell lines and observed VCM to be chondrotoxic. They investigated cytotoxicity using a lactate dehydrogenase (LDH) assay [6]. However, the LDH assay was developed as a guide when the direct isolation of cells is not achieved in high-density attached cells. Therefore, the metabolite level is measured by the amount of LDH released into the cell-culture supernatant and taken as the quantitative value for the loss of live cells. In other words, the measurements are not performed directly in cells but through the reduction of enzymes released by cells in the medium. The data would have been more robust if metabolic activity and DNA synthesis, which are used in a single-course culture method, had been used instead of spectrophotometric analysis of the reduction from pyruvate to lactate [28,29].

The most popular cytotoxicity analysis performed in single-course cell cultures involves the colorimetric measurement of cell proliferation using MTT tetrazolium salt. The MTT method has been in use since 1983 for cell proliferation, viability, and toxicity identification [30,31].

This method is quite sensitive in the quantification of cell proliferation and cytotoxicity, especially in chemotherapeutic drugs, in which only live cells with metabolic activity reduce tetrazolium salt to colored and insoluble formazan. Therefore, this method identifies only live cells. It not only identifies cell viability and cytotoxicity but also identifies cell activation and proliferation. Tetrazolium salts have been proven in the literature as the most sensitive and most soluble methods for accurate cell vitality, toxicity, and proliferation analyses, and several new methods, such as WST, XTT, MTS, and MTT, have been developed [28—32].

Toxicity studies are generally performed on tissues from animal articular cartilage. Obviously, human and animal tissues have different sensitivities, and thus, results may be different and misleading [24]. The use of a single type of cell in cell-line systems, the lack of complicated coordination mechanisms in between cells and the micro frame, and the inhibition of interactions with the extracellular matrix structure make in vitro tests problematic to adopt to in vivo conditions, in terms of compatibility [33]. The biggest disadvantage of cell lines is that they do not carry all of the genotypical and phenotypical features of the original structure because they are genetically modified [34].

In the present study, human primary chondrocyte cultures were used to investigate the chondrotoxicity of three agents. Although the experiment was in vitro, cell cultures were not obtained from animals, and a cell line was not used. Therefore, we believe that the findings are robust. Considering the MBC reference values, VCM (16 mg/L), TEIC (64 mg/L), and LZD (32 mg/L) have no chondrotoxic effects when administered locally. The present study observed that these antibacterial agents, which are frequently used locally in orthopedic infections, are not toxic to cartilage, especially in terms of viability and proliferation.

Conclusion

We believe that these antibacterial agents, which showed no chondrotoxicity in either a cellular dimension or on a molecular level, can safely be locally used in orthopedic surgery, especially against MRSA agents. However, this is only an in vitro study. Antibiotics did not show adverse effects on cultured chondrocytes from six patients in the age range of 59—71 years. We cannot conclude that the antibiotics are safe in vivo in orthopedic surgery. The evidence supports the hypothesis only, and it does not disprove the hypothesis. In an extension of this study, the effect of the antibiotics on cell function should be performed in different phases of growth and for variable lengths of exposure time. Further in vivo molecular studies investigating these agents comparatively and in a cellular dimension with larger samples are warranted.

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Competing interests

None declared.

Ethical approval

Not required.

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